



Egg serpins: The chicken and/or the egg dilemma

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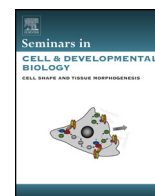
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Review

Egg serpins: The chicken and/or the egg dilemma



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ABSTRACT

Twenty-seven serpins belonging to clade A, B, C, D, E, F, G, H and I serpins are currently referenced in chicken genome databases. Phylogenetic analysis of chicken serpins revealed that ovalbumin (Serpinsb14) and its paralogs ovalbumin-related protein Y (Serpinsb14b) and ovalbumin-related protein X (Serpinsb14c) are found in bird species. These clade B serpins are specifically expressed in reproductive tissues and exported in the egg where they constitute major protein components. These data suggest that these three paralogs have probably appeared in birds to face new environments and ensure the extra-uterine development of an embryo in a shell egg. Twelve other serpins have been identified in the newly produced egg, some of them having a specific distribution in the respective egg structures (eggshell, egg white, vitelline membrane and egg yolk). The physiological role of these egg serpins remain largely unexplored, but there is increasing evidence in literature or by homologies with their mammalian counterparts, that some of them participate in cell proliferation, tissue remodeling and/or angiogenesis associated with folliculogenesis and development of extraembryonic structures, eggshell biomineralization, egg defense and nutrition of the embryo. A better knowledge of the phylogenetic evolution of these 15 serpins in other oviparous species, on their egg distribution, on their regulation during embryonic development (activation/degradation/transfer) and on their functional specificity, is needed to better appreciate their role and their bird-specificity. These review shed light on the multiple possibilities that offer the avian egg model to study the role of serpins in reproduction and developmental biology.

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Abbreviations: ACC, amorphous calcium carbonate; CAM, chorioallantoic membrane; ESM, eggshell membrane; OVAX, ovalbumin-related protein X; OVAY, ovalbumin-related protein Y.

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1. Evolution and distribution of egg serpins

Systematic analysis of National Center for Biotechnology Information and chicken Ensembl databases for identifying serpins in chicken species revealed the presence of 27 members of this family (Table 1, Fig. 1). Among these, 16 serpins are still predicted and have been referenced in databases by automated computational analysis of genomic chicken sequences annotated using gene prediction methods, which sometimes can lead to discrepancies between gene and protein names (as an example, *Serpina2* gene corresponds to a predicted Serpinb10 protein, Table 1). However, the relevance of 5 of them have been recently validated, as serpins a8, c1, d1 and f1 were unambiguously identified in the egg (see §1.2). Thus, 11 of the serpins identified to date from genome analysis, need further validation and the information available about their physiological functions is therefore very partial, even inexistent. Surprisingly enough, *Serpine1* was not identified in this analysis. To investigate whether *Serpine1* exists as a pseudogene in chicken or whether it was not annotated correctly in databases, *Serpine1* was searched in the chicken genome using reciprocal tBLASTn. The serpin was not found and the closest gene identified by reciprocal tBLASTn against human genome referred to *SERPINE2*, which was actually identified in both Pubmed and Ensembl databases and referenced in Table 1. Thus, to date, we cannot ascertain that this gene is absent due to errors in genome annotation or whether it actually disappeared in chickens during evolution. Chicken ov-serpins are largely represented as 10 clade B serpins could be identified [1,2]. These ov-serpins are clustered on a 150 kb locus chromosome 2q (Fig. 1B) and comprise *Serpina1*, *Serpina2*, *Serpina5*, *Serpina6*, two *Serpina10* homologs (*Serpina10*, *Serpina10b/MENT*), *Serpina12* and *Serpina14*, *Serpina14b* and *Serpina14c* namely ovalbumin and its related genes Y (OVAY) and X (OVAX). Another cluster on chromosome 5 was identified containing 7 members of the *Serpina* family (Fig. 1E). This cluster includes 5 homologs of alpha1-antitrypsin/alpha1-proteinase inhibitors, *Serpina1*, *Serpina3*, *Serpina4*, *Serpina5*, *Serpina9*, which correspond to human antitrypsin, alpha1-antichymotrypsin, kallistatin, Protein C inhibitor, and *SERPINA9*, respectively. Out of these 27 serpins, only 15 are actually recovered in the chicken egg in which the biological significance and biological activity intimately depends on the process of egg formation and on their subsequent localization (eggshell/egg white/vitelline membrane/yolk). This first part will review the evolution of these chicken serpins in vertebrate species and their distribution in the various egg compartments.

1.1. Phylogenetic analysis of chicken serpins

In serpin genes, some have shown a strong correlation between genomic organization, patterns of amino acids at specific sites, and insertion/deletion patterns, which contributed to identify serpin groups and to decipher vertebrate serpin evolution [3,4]. Serpin genes have rapidly evolved; a high sequence divergence is found between all serpin clades, the sequence identity varying from 22% to 29%.

Phylogenetic analysis of the 27 serpins found in the chicken genome shows that serpin genes have been originated and dupli-

cated before the divergence of teleosts. Another duplication event occurred after divergence between species, for example, the clade A of *Gallus gallus* encompasses seven *Serpina* genes with a sequence identity around 47%. The same phenomenon is observed for mammal and fish.

Using the phylogenetic trees available in Ensembl (<http://www.ensembl.org>), and because of the stringency of the method used, three serpins groups are distinguished. The first phylogenetic tree contains serpins from clade B, C, E and I (Fig. 2), the second refers to serpins from clade A, D, F, G and H (Fig. 3), and the last tree, contains only one clade A serpin, *Serpina8*.

The clade B serpin, present in the first tree, contains ovalbumin gene (*Serpina14*) and its recently duplicated OVAY (*Serpina14b*) and OVAX (*Serpina14c*) [5–7]. OVAX, is not annotated in Ensembl but by using reciprocal tBLASTn alignment method (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), this gene could be easily identified as the closest neighbor of OVAY in chromosome 2 with a 73% of protein sequence identity as previously shown [1,2]. The evolution of clade B serpins starts before the split of bony fishes and tetrapods, 450 millions years ago, leading to at least six clade B serpin genes found in mammal and bird genomes. The *G. gallus* genome contains ten clade B serpin genes on chromosome 2 in the same syntenic locus (Figs. 1 and 2). Six of them could also be found in *Homo sapiens* genome (*SERPINA1*, *B2*, *B5*, *B6*, *B10*, *B12*). Chicken clade B serpins (Figs. 1 and 2) are syntenic with two loci on chromosome 6 and 18 in human and other mammalian genomes, which suggests a split resulting to a break of synteny in mammals [2]. Recent duplications events occurred independently in fishes, mammals and birds, after divergence between these species. Due to recent duplication, avian *Serpina14* (ovalbumin), *Serpina14b* (OVAY) and *Serpina14c* (OVAX), have no human or other mammalian species orthologues and seem to be specific to oviparous species [1,8,9]. Three orthologues to ovalbumin are referenced in duck, flycatcher and turkey in Ensembl database but information is lacking for other oviparous species. However, it is noteworthy that we found a potential orthologous of *Serpina14c* in *Alligator mississippiensis* (A0A0Q3ZV65), sharing 56% protein sequence identity with the chicken homolog. These ovalbumin genes adapted to oviparous species, are supposed to have lost their protease inhibitor activity [1], and potentially acquired specific properties adapted to the development of an embryo in an egg exposed to terrestrial environments [8,9].

Clade A, D, F, G and H serpins are found in the second phylogenetic tree. As shown in Fig. 3, except clade A serpins, each of these serpins has an orthologue in both *G. gallus* and *Bos taurus*.

Similarly to clade B serpins, and for both species (*G. gallus*; *B. taurus*), duplication events led to the presence of several clade A serpins after species divergence (Fig. 4). Concerning *G. Gallus* genome, this duplication gave rise to *Serpina1*, *a3*, *a4*, *a5*, *a9*, *a10* and *a12*. These serpins have similar peptidase inhibitor function and are essentially expressed by the liver. The synteny analysis (<http://www.genomicus.biologie.ens.fr/>) reveals that clade A genes are localized in a gene cluster in chicken chromosome 5 (Fig. 3). The same phenomenon is observed in *B. Taurus*, where all clade A serpins are localized in a unique gene cluster on chromosome 21. Clade A serpins show multiple recent duplications leading to ten

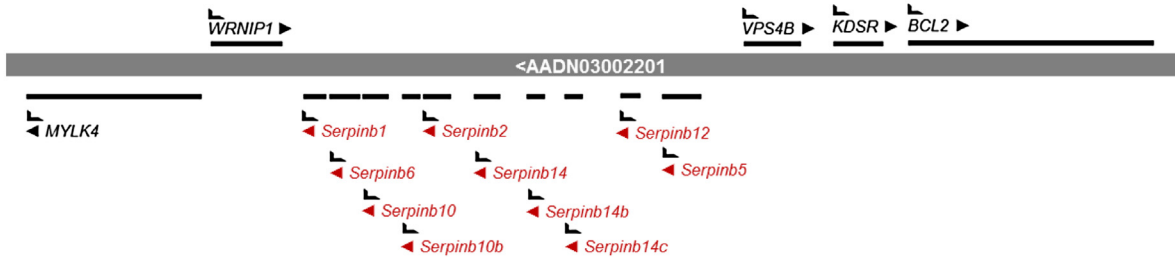
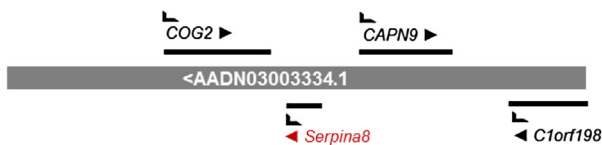
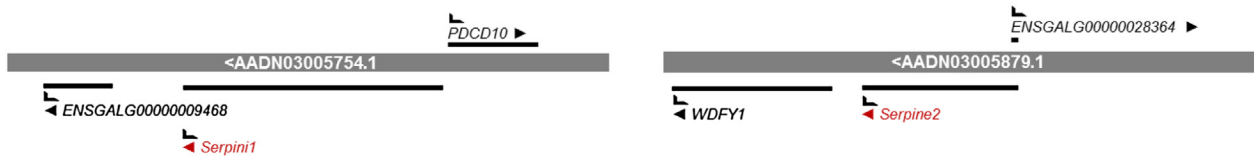
A. Chromosome 1: 169,314,156-169,437,377/194,273,709-194,367,705**B. Chromosome 2: 67,605,721-68,022,782-Chicken Clade B serpins****C. Chromosome 3: 39,710,086-639,884,651****D. Chromosome 8: 7,311,755-7,430,238****F. Chromosome 9: 7,770,814-7,860,071/20,010,493-20,102,119****E. Chromosome 5: 44,767,283-44,957,645****G. Chromosome 15: 23,698-165,087****H. Chromosome 19: 5,378,317-5,465,204**

Fig. 1. Chromosome localization of chicken serpins. Serpins and flanking genes with their respective orientation (backward/forward) are drawn to scale. Assignment of gene names was established based on Ensembl database information or by comparison with human orthologs. The name *MENT* (Gene ID: 1017749622) has been replaced by *Serpina10* in accordance with [1], based on the high sequence identity of *Serpina10* and *Serpina10b*, suggesting that they are paralogs. Accession numbers are indicated in Table 1. With the exception of *Serpina14c*, all genes were found in Ensembl database.

Table 1

Chicken serpins. Analysis of Pubmed database (<http://www.ncbi.nlm.nih.gov/pubmed/>) using “serpin” and “gallus” as key words in “Protein” database resulted in 93 proteins hits; whereas using “serpin” to search related proteins in Chicken Ensembl database (<http://www.ensembl.org/Gallus.gallus/>) gave 83 hits (01-18-2016). Manual compilation and integration of results by removing redundancy to keep only gene IDs resulted in a total of 27 distinct serpin genes.

Protein Name [Gallus gallus]	Protein accession number	Gene symbol	Gene ID	Gene accession number
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 precursor	NP.001264422.1	<i>Serpina1</i>	423434	ENSGALG00000023070
PREDICTED: protein Z-dependent protease inhibitor	XP.015143253.1	<i>Serpina10</i>	423432	ENSGALG00000020391
PREDICTED: serpin A3-4-like isoform X1	XP.015143255.1	<i>Serpina12</i>	107049127	ENSGALG00000028598
PREDICTED: alpha-1-antiproteinase	XP.015143260.1	<i>Serpina3</i>	772339	ENSGALG00000020388
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 precursor	NP.001264421.1	<i>Serpina4</i>	423433	ENSGALG00000010969
PREDICTED: alpha-1-antitrypsin isoform X1	XP.421344.1	<i>Serpina5</i>	423435	ENSGALG00000020390
PREDICTED: angiotensinogen	XP.004935550.1	<i>Serpina8</i>	421543	ENSGALG00000011117
PREDICTED: alpha-1-antitrypsin	XP.004941955.1	<i>Serpina9</i>	423436	ENSGALG00000020389
PREDICTED: leukocyte elastase inhibitor isoform X1	XP.015137679.1	<i>Serpinb1</i>	420894	ENSGALG00000019555
PREDICTED: heterochromatin-associated protein MENT	XP.004939740.1	<i>Serpinb10</i>	101749622	ENSGALG00000019554
heterochromatin-associated protein MENT	O73790.1	<i>Serpinb10b</i>	395715	ENSGALG00000019553
PREDICTED: serpin B12	XP.418985.2	<i>Serpinb12</i>	420899	ENSGALG00000012872
ovalbumin	AAB59956.1	<i>Serpinb14</i>	396058	ENSGALG00000012869
ovalbumin-related Y	NP.001026172	<i>Serpinb14b</i>	420897	ENSGALG00000019551
ovalbumin-related protein X	AGN32861.1	<i>Serpinb14c</i>	420898	NM.001276386.1
PREDICTED: serpin B10	XP.418982.1	<i>Serpinb2</i>	420896	ENSGALG00000019552
PREDICTED: serpin B5	XP.418986.3	<i>Serpinb5</i>	420900	ENSGALG00000012873
serpin B6	NP.001006377.1	<i>Serpinb6</i>	420895	ENSGALG00000012866
PREDICTED: antithrombin-III	XP.422282.3	<i>Serpinc1</i>	424440	ENSGALG00000004591
PREDICTED: heparin cofactor 2	XP.001232767.1	<i>Serpinb1</i>	395877	ENSGALG00000001396
SERPINE2 serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	E1BWU2	<i>Serpine2</i>	424805	ENSGALG00000005135
PREDICTED: serpin E3	XP.015131346.1	<i>Serpine3</i>	418875	ENSGALG00000017017
PREDICTED: pigment epithelium-derived factor isoform X1	NP.001244218.1	<i>Serpinf1</i>	417561	ENSGALG00000003015
serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	XP.015151529.1	<i>Serpinf2</i>	100857105	ENSGALG00000002987
plasma protease C1 Inhibitor Precursor	XP.003641424.1	<i>Serping1</i>	423132	ENSGALG00000007381
PREDICTED: serpin H1 isoform X1	XP.015136453.1	<i>Serpinh1</i>	396228	ENSGALG000000011214
neuroserpin precursor	NP.001004411.1	<i>Serpinh1</i>	425002	ENSGALG00000009470

Serpina (*Serpina1*, *a3*, *a4*, *a5*, *a6*, *a7*, *a10*, *a11*, *a12*, *a14*). Six of them belong to the subgroup of *Serpina3* (*Serpina 3-1*, *a3-5*, *a3-6*, *a3-7*, *a3-7-like*, *a3-8*), and share almost 70% of identity with human SERPINA3 members [10]. The synteny analysis of clade A serpins for *B. taurus* shows the presence of a gene cluster in chromosome 21, similar to the cluster on chromosome 5 found in *G. gallus*. In both *G. gallus* and *B. taurus* loci, serpins from clade A are localized in the same syntenic locus.

In the third phylogenetic tree, only *S. Serpina8* is present and could be found in all species. In contrast to the recent publication where authors used synteny and signature sequences to analyse *Serpina8* gene in other species [11], Ensembl phylogenetic tools reveals that the duplication of *Serpina8* occurred before divergence of teleosts. This serpin has also diverged rapidly as opposed to the other serpins. Compared with other clade A inhibitory serpins, this SERPINA8 also named angiotensinogen has a very specific role in vertebrates, in that it is proteolytically processed by renin to generate angiotensin I, which is further trimmed into vasoactive angiotensin II that regulates blood pressure.

To conclude, serpins have been duplicated before the divergence of teleostean giving rise to 16 clades (from A to P) [12,13]. Nine clades are present in the avian genome and two of them (clade A and B) have been recently duplicated leading, among others, to ovalbumin, OVAX and OVAY, which are specific to bird species. A better understanding of the function of these proteins is necessary to highlight the reason of their duplication and specificity.

1.2. Tissue distribution in chicken (the chicken and/or the egg question)

There is very few information on the expression and biological activities of chicken serpins. Chickens, by homology with mam-

mals are expected to express serpins at a basal state to ensure basic biological processes such as coagulation/hemostasis (PREDICTED: angiotensinogen, Plasma Protease C1 Inhibitor Precursor, PREDICTED: antithrombin-III, PREDICTED: heparin cofactor 2), cell proliferation (SERPINB5) although controversial [14], inflammation (PREDICTED: leukocyte elastase inhibitor), etc. At sexual maturity of the pullets, the secretion of ovarian steroid hormones by the theca of the growing follicle triggers the development and differentiation of the hen oviduct. This development is concomitant with the formation of the first egg that will contain nutrients and bioactive proteins to support embryonic development [15]. The formation of chicken egg is a spatial and temporal process that relies on the ovary (site of sex steroid synthesis, gametogenesis and yolk formation) and the oviduct, which receipts the ovulated mature yolk and where the white, the shell membranes and the shell are successively deposited in very specialized regions, the magnum, the isthmus and the uterus/vagina, respectively (Fig. 5) [15]. Analyses and integration of the various proteomic data published on the cuticle/eggshell, egg white, vitelline membrane and egg yolk revealed the presence of 15 serpins in the freshly laid egg [16–28] (Fig. 5). The eggshell contains 14 different serpins, with Serpinb6 and Serpinb1 being specifically found in this compartment. The egg white recovers 6 serpins including one which has been identified only in this compartment (Serpinb5). The vitelline membrane possesses 5 serpins which are also components of the egg white and the eggshell, and the egg yolk contains 10 serpins that are also listed in the other egg compartments (Fig. 5). As previously discussed, 3 of these egg serpins (Serpinb14, Serpinb14b, Serpinb14c) might be specifically associated with bird species and the extra-uterine development of the embryo within an egg.

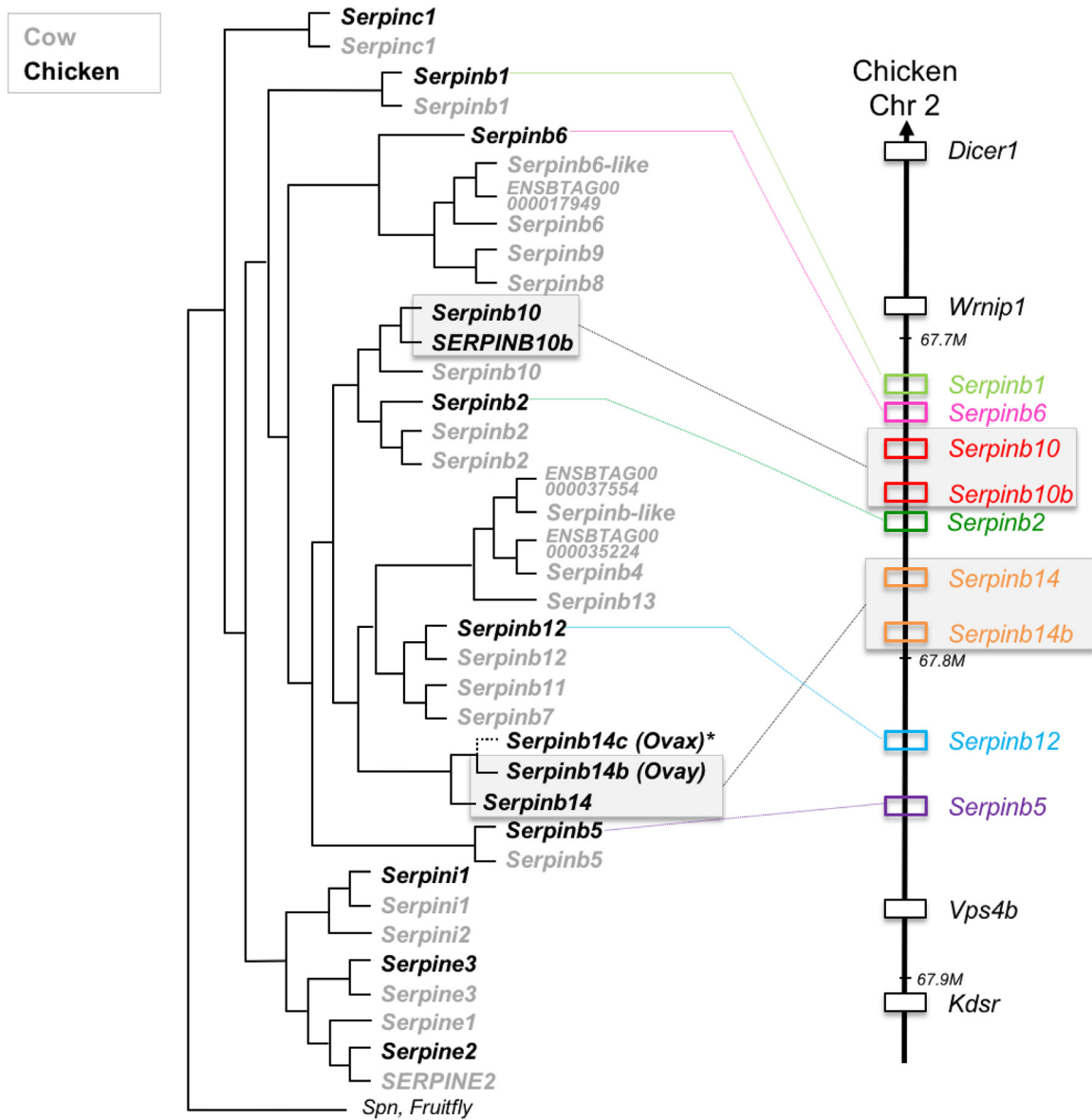


Fig. 2. Phylogenetic tree of SERPINB, C, E and I and loci representation. Phylogenetic tree, generated by Ensembl, merging maximum likelihood and neighbor joining trees, shows serpins orthologs found for chicken and cow. All *G. gallus* clade B serpins are localized in the same gene cluster on chromosome 2.

*The *Serpinb14c*, not annotated in Ensembl was manually added to this figure. The protein sequences of the 27 serpins genes found in the chicken gene genome were aligned and a serpin phylogenetic tree was generated (using the website <http://www.phylogeny.fr>), in order to compare validate the position of *Serpinb14c* in the phylogenetic tree.

2. Egg serpins: an update

With the exception of ovalbumin (*Serpinb14*) and its related protein X (*Serpinb14c*) and Y (*Serpinb14b*), most egg serpins recovered in the egg are not specifically expressed to support egg formation. Therefore, to appreciate their respective physiological activity in egg, it is important to have a good representation of the process of egg formation in mind, as their function is intimately linked to their localization within the egg.

2.1. Biological significance of egg yolk serpins

Major proteins of the egg yolk, with the exception of immunoglobulins, are synthesized by the liver of laying hens in which protein synthesis and lipogenesis are stimulated 15–20 fold at sexual maturity. Egg yolk proteins result from the stimulation of hepatic expression of preexisting proteins and neo synthesis of specific egg components. Once secreted into the blood, egg yolk

precursors such as very-low density lipoproteins are transported to the ovarian follicle and incorporated in the growing yolk follicles, via receptor-mediated endocytosis [29]. Meanwhile, the liver continues to express many proteins which are not related to vitellogenesis and which can be unselectively incorporated into the egg yolk by passive binding to egg yolk-specific proteins. Eleven serpins have been identified in the egg yolk: *Serpina1* (alpha1-antitrypsin), *Serpina4* (kallistatin), *Serpina8* (angiotensinogen), *Serpinb14* (ovalbumin), *Serpinb14b* (OVAY), *Serpinb14c* (OVAX), *Serpinc1* (antithrombin III), *Serpind1* (heparin cofactor II), *Serping1* (plasma Protease C1 Inhibitor), *Serpinf1* (pigment epithelium-derived factor) and *Serpinf2* (alpha2-antiplasmin) [25,27]. Except *Serpina1*, *Serpinb14*, *Serpinb14b*, *Serpinb14c*, serpins identified in egg yolk are expressed by the chicken liver, regardless of the sexual maturity of the hens [30], corroborating that these serpins are not specifically expressed to support vitellogenesis. *Serpina1*, *Serpinb14*, *Serpinb14b*, *Serpinb14c* might be expressed within the ovary by surrounding cells including granulosa cells and theca to

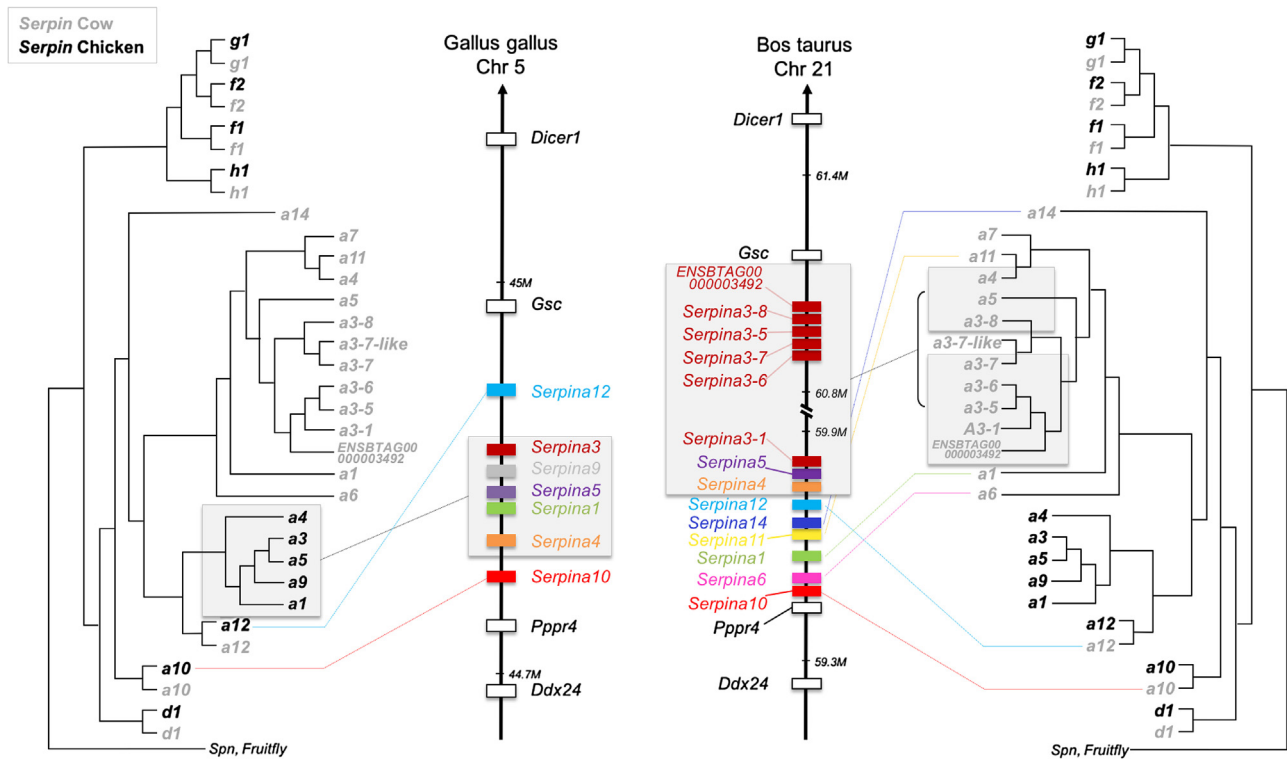


Fig. 3. Comparative analyses of *Gallus gallus* and *Bos Taurus* loci including clade A, D, F, G, and H serpins. Phylogenetic trees are generated by Ensembl. Clade A serpins are found in the same syntenic cluster in both *G. gallus* and *B. taurus*. The *B. taurus* genome was preferred for this study as it is well referenced for this locus. Moreover, serpins from clade B found in *B. taurus* genome possess more duplications (as chicken Serpinb14 for example) as compared with human genome. The phylogenetic trees were processed by ENSEMBL, with the complete sequences including amino- and carboxy- terminal extensions.

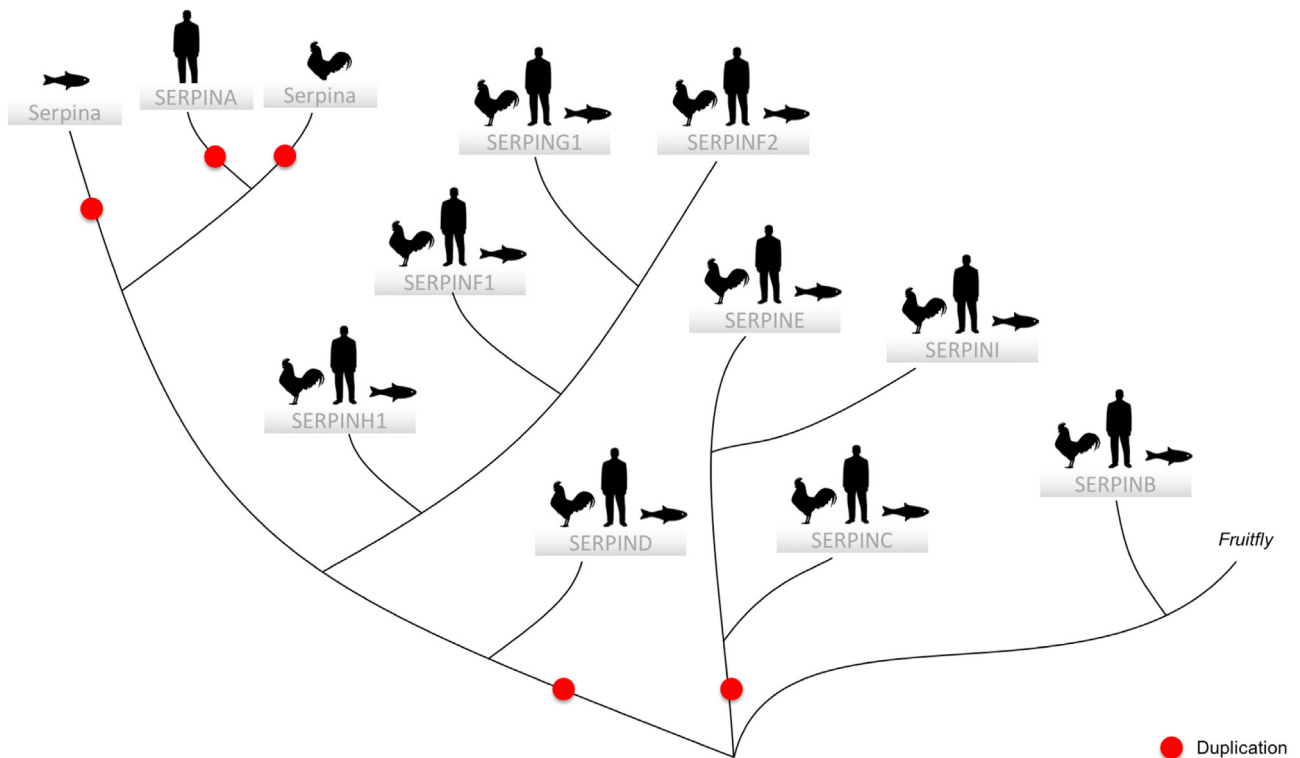


Fig. 4. Evolutionary scenario of SERPIN from clade A, D, F, G and H for mammal, birds and fishes. Last SERPINA duplication appears, for mammals, birds, and fishes, after divergence between species. Speciation branches are represented: for example, for SERPING1 and SERPINF2 the node represents the last common ancestor, and the divergence before speciation between different species. The red dot represents paralog duplication after divergence between species. This phylogenetic tree was made using PRANK (<http://www.ebi.ac.uk/goldman-srv/prank/>) to align sequences and RAxML (<http://sco.h-its.org/exelixis/software.html>) to build the tree.

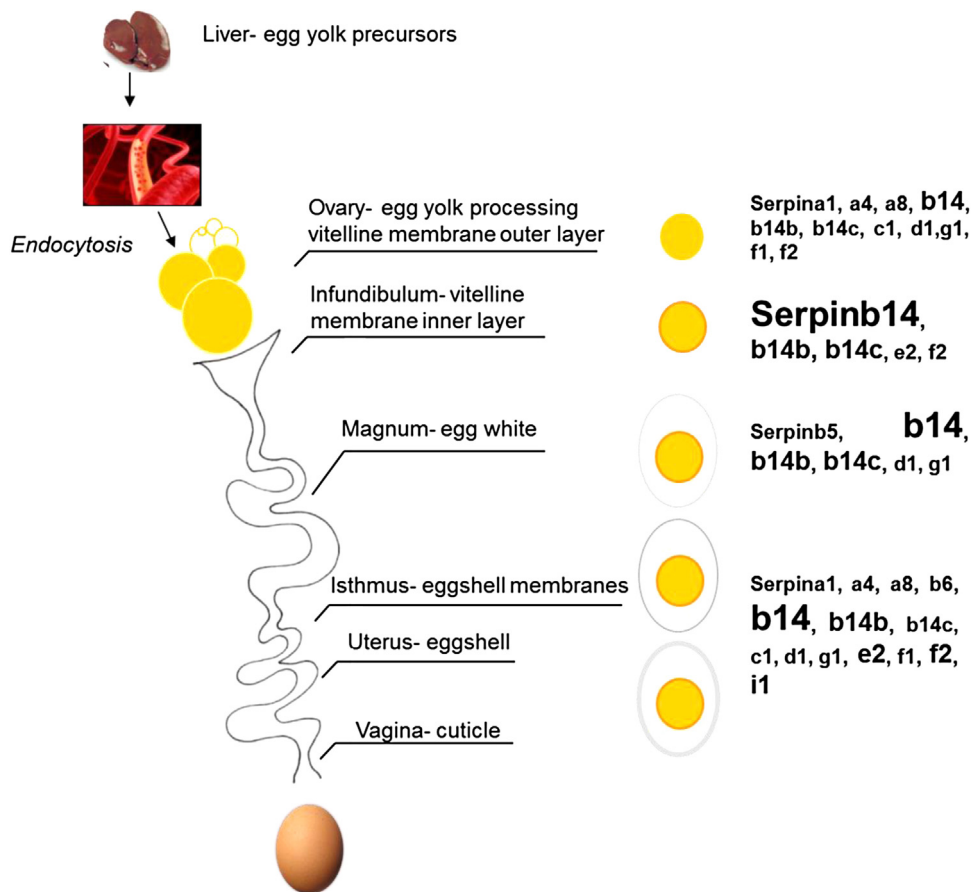


Fig. 5. Schematic representation of egg formation and serpins identified in each egg compartments. For each compartment, the size of the font roughly indicates the relative quantity of one serpin to another. Bigger letter corresponds to abundant to very abundant proteins, smaller letters correspond to low abundant proteins and normal letters to proteins with intermediate abundance. Note that compartments are not comparable, as the relative quantity intimately depends on the intrinsic composition of each compartment. Data were extracted from [25,27] (egg yolk), [26] (vitelline membrane), [22,28] (egg white), and [23,80] (eggshell).

be incorporated in the yolk, or be recovered in the egg yolk by passive diffusion from the egg white where they are major components. Actually, the exhaustive analysis of serpins expression in chicken male or female tissues has never been investigated and in females, except for Serpinb14, Serpinb14b and Serpinb14c, their expression does not seem to be hormone-regulated [30]. The functional annotation of egg yolk proteins has revealed that serpins identified in the egg yolk are essentially known actors of coagulation/fibrinolysis cascades [24]. This consideration, together with their very low abundance in egg yolk, questions the biological relevance of these eleven serpins in the egg yolk.

2.2. Vitelline membrane serpins: a predicted role in folliculogenesis, defense and angiogenesis

The vitelline membrane is the acellular proteinaceous membrane at the interface between the egg white and the egg yolk. This membrane is composed of two distinct layers (outer and inner layers) separated by a thin continuous membrane [31]. The inner layer, in contact with the yolk and the oocyte and corresponding to the zona pellucida in mammals, is constituted of interlaced proteinaceous fibers most likely produced by granulosa cells and/or liver cells, during vitellogenesis/folliculogenesis. The outer layer contains proteins forming a lattice network of fine fibrils, secreted by the infundibulum (Fig. 5). Fertilization of the oocyte by a sperm cell occurs in the infundibulum, presumably prior to the secretion of the vitelline membrane outer layer. The vitelline membrane has different roles in avian reproduction. The inner layer encloses zona

pellucida proteins known for their role in sperm-egg interaction during fertilization [32]. The inner surface of vitelline membrane promotes cell growth [33] following egg fertilization, which in turn progressively degrades the vitelline membrane to form a vascularized tissue around the yolk, namely the yolk sac [34]. The vitelline membrane acts as a natural filter barrier to separate egg white from yolk components, and to prevent microbial contamination of yolk potentially coming from the albumen. The outer layer of the vitelline membrane is also rich in antimicrobial proteins (lysozyme, ovotransferrin, avian beta defensin 11). Proteomic analysis of hen egg vitelline membrane revealed the presence of 137 proteins [26] including 5 serpins: Serpinb14 (ovalbumin), Serpinb14b (OVAY), Serpinb14c (OVAX), Serpine2 (glia-derived nexin/protease nexin-1) and Serpinf2 (alpha2-antiplasmin). The distribution of these avian serpins within the various layers of the vitelline membrane remains unknown to date and their biological roles are still unclear. As previously mentioned, Serpinb14, Serpinb14b and Serpinb14c are egg-specific proteins. Their role within the egg and the vitelline membrane still remains elusive although they constitute major components of this compartment [26] (Fig. 5). No protease-inhibiting activity has been found for these serpins to date. A recent study demonstrated that Serpinb14c possesses heparin-binding properties (Fig. 6) and antibacterial activities, in contrast to its homolog Serpinb14 [35]. Serpinb14c might therefore contribute to egg defense together with other active antimicrobials present in the vitelline membrane.

By comparison with their mammalian homologs, avian Serpine2 and Serpinf2 are presumed to have important functions

in egg formation, especially during follicular development and/or ovulation phases. Both serpins are expressed in bovine granulosa cells [36,37] and temporal expression of Serpine2 gene can be observed during follicular growth: high expression level is indeed associated with large growing follicles whereas lower expression is rather observed in small growing follicles or in pre-ovulatory follicles [36]. Plasmin, the cognate serine protease of Serpine2 and Serpinf2 [38,39], is involved in the extensive remodeling of the follicular connective tissue and degradation of the basal lamina during follicular expansion. Ovulation also involves proteolytic events at the follicular apex responsible for the formation of the stigma and the release of the mature oocyte. Plasminogen activators/plasmin system is believed to participate in follicular maturation in chickens [40]. Serpine2 possesses glycosaminoglycan-binding properties [38] and knowing that there are some pre-ovulatory changes of glycosaminoglycans content (i.e. degradation) in the stigma, the area of the ovarian surface where the mature chicken follicle will burst through during ovulation [41], glycosaminoglycans produced by granulosa cells and their degradation might regulate the proteolytic processes during the follicular growth and/or the ovulation, via the interaction with follicular serpins and the modulation of their activity [42].

Besides follicular growth, the vitelline membrane also supports cell proliferation and angiogenesis associated with embryonic development. In chicken, Haas and Spratt have reported that components of the inner membrane promote the outgrowth of extraembryonic tissues onto the vitelline membrane [33]. Serpins from vitelline membrane might participate in these processes via both antiprotease-dependent and independent mechanisms. SERPINE2 is known to interact with several modulators of angiogenesis, such as proteases (thrombin, plasmin, plasminogen activators), extracellular matrix proteins and glycosaminoglycans [43,44]. Antiangiogenic properties have been reported *in vitro* and *in vivo* for SERPINE2, which has been demonstrated to inhibit vascular epithelial growth factor activity on endothelial cells (including proliferation and migration) and to decrease cell spreading on vitronectin [45]. Interestingly, its anti-angiogenic effects do not involve its inhibitory site but rather rely on its glycosaminoglycan-binding properties [45]. Serpine2 might therefore regulate angiogenesis which guides the development of yolk sac by targeting pivotal cell signaling pathways such as the Hedgehog pathway [46].

2.3. Egg white serpins: a role in nutrition and defense. What else?

Egg white is an aqueous solution mainly composed of water (88%), proteins (90% dry matter), minerals (6% dry matter) and free glucose (3.5% dry matter). The dominant physiological role of egg white is assumed to provide nutrients for the embryo and protection against microbial contamination. Clade B serpins including ovalbumin (Serpib14), OVAY (Serpib14b) and OVAX (Serpib14c) are major components of the egg white [28], with ovalbumin accounting for 54% of egg white proteins (about 50 mg/mL). All three proteins are mainly produced by the oviduct and more specifically by tubular gland cells of the chicken's magnum, responsible for egg white formation [8] (Fig. 5). The additional major proteins found in this compartment are lysozyme, ovotransferrin, which both prevent bacterial proliferation and dissemination. Egg white is also characterized by the presence of numerous active protease inhibitors including ovomucoid and ovoinhibitor (Kazal-like proteins), cystatin and ovostatin [47], which are assumed to protect egg white proteins from inappropriate/early proteolytic events. Non-inhibitory properties of certain serpins including ovalbumin, ovalbumin-related protein X or maspin (Serpib5) can be possibly explained by multiple devi-

ations in the hinge region of the reactive center loop, compared with the consensus sequence for inhibitory serpins [1] (Fig. 6). The physiological function of all three paralogs is still unclear. The on-going hypothesis is that it would serve as a source of amino-acids for the developing chicken embryo from the eleventh day of incubation while the egg white migrates to the amniotic fluid to be orally absorbed by the embryo. Up to that stage, egg white proteins are probably protected from proteolysis thanks to major egg white active antiproteases. Indeed, egg white proteins remain essentially uncleaved during the first half incubation, as revealed by proteomic approaches [48]. It is noteworthy that Serpinb14 naturally undergoes some conformational changes during egg incubation, to convert to a heat-stable form named S-ovalbumin [49]. This S-ovalbumin is characterized by chemical inversions of serine residues into the D configuration, and other subtle changes, that are supposed to give a thermodynamic advantage to the structural stability of S-ovalbumin [50]. Interestingly, once swallowed from the amniotic fluid, ovalbumin does not seem to be fully altered in the gastrointestinal tract of the embryo [49]. Ovalbumin is recovered in the extracts of many embryonic organs including the head, eye, heart, liver, intestine, spinal cord, muscle, dermis, and bone [49]. Surprisingly enough, the presence of uncleaved ovalbumin persists in embryonic organs suggesting that at least a fraction of ovalbumin molecules could be transported intact to embryonic organs [49]. This observation together with the absence of ovalbumin mRNA expression in these organs and with the fact that the neonate organs are no longer positive for ovalbumin shortly after hatching [49], suggests that egg white ovalbumin may not merely serve as a source of amino acid but may also have a more active/direct function on developing tissues.

With regard to OVAY and OVAX, there is no information available about their susceptibility to convert to a S-form, similarly to ovalbumin, nor about their presence in embryonic tissues during incubation. But, the abundance of OVAY in egg white was shown to be significantly affected during incubation [48]. Its predicted Lys-His reactive site suggests that OVAY could inhibit trypsin-like proteases [1] and thus possibly gastrointestinal proteases of the embryo and/or yolk proteases. Considering OVAX, it was shown to lack inhibitory activity against trypsin/chymotrypsin-like proteases [35]. However, it exhibits antimicrobial activities against two pathogens, *Listeria monocytogenes* and *Salmonella enterica* Enteritidis via its heparin-binding domain [35]. These activities suggest a role for OVAX at least in innate defense. Similar function has been proposed for mammalian heparin-binding serpins including Heparin cofactor II/SERPIND1 [51], which is also present in the egg white. The expression of Serpin1 and Serpin1 in the oviduct and more particularly in the magnum has not been investigated yet. Their presence in the egg white could be the consequence of oviducal expression but could also result from passive diffusion from the yolk. Concerning Serpinb5, its role in the egg white will not be further discussed since it was identified as very minor component [52].

2.4. Eggshell serpins as regulators of biomineralization process

The calcified chicken eggshell is a natural envelope which protects the developing embryo from physical and microbial assaults. It is composed of 95% calcium carbonate (calcite polymorph), 1.5% water and 3.5% proteins, polysaccharides and proteoglycans [53]. Avian eggshell is a porous mineral layer with a well-defined structural polycrystalline organization (Fig. 7A). Biomineralization may be defined as the production of the hard tissue characterized by a specific minerals/organic matrix framework, by a living organism. Eggshell proteins and proteoglycans play a key role in shell forma-

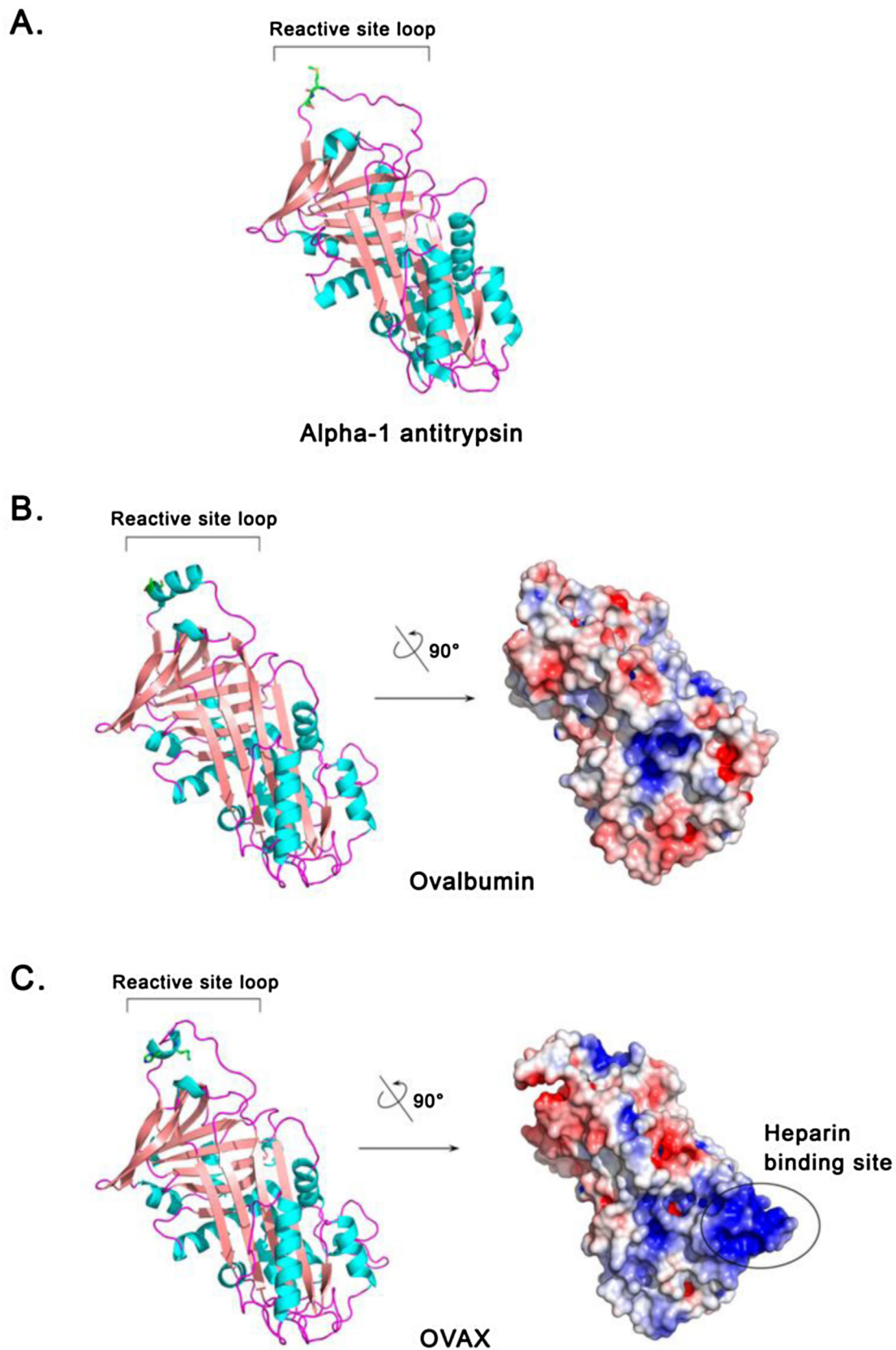


Fig. 6. Three-dimensional structures of clade A and clade B serpins.

(A) Cartoon representation of human alpha-1 antitrypsin/SerpinaA1 (1QLP) showing the exposed reactive site loop that interacts with the protease active site *via* the P1-P1' residues (Met358-Ser359) to form a Michaelis complex. This loop is further cleaved by the protease and leads to the formation of an irreversible, covalent complex between the serpin and its cognate protease. (B and C) 3D structures of ovalbumin/SerpinaB14 and OVAX/SerpinaB14c, respectively. Although the structures of ovalbumin and ovalbumin-related protein X are highly similar to that of known inhibitory serpins, their reactive site loop are unlikely to interact with proteases [35,81], which can be explained by multiple deviations in the hinge region of the reactive center loop, compared with the consensus sequence for inhibitory serpins [1]. The solvent-accessible

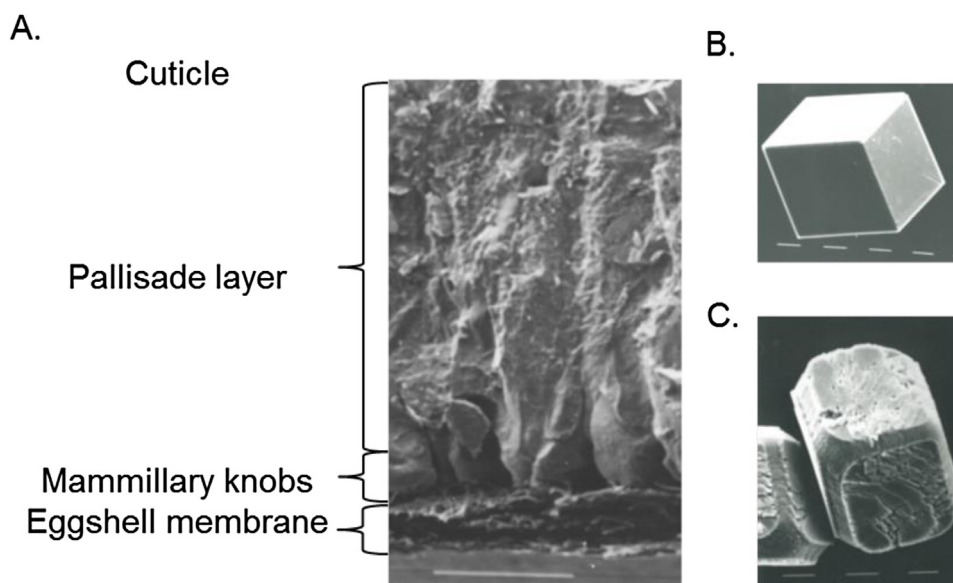


Fig. 7. Structure of the eggshell and effect of ovalbumin on calcium carbonate crystals. (A) Ultrastructural structure of chicken eggshell (scanning electron microscopy). (B) Control crystal. (C) Crystal obtained in the presence of ovalbumin (133 $\mu\text{g/mL}$). The morphology and the size of crystals (half reduction) are significantly modified in the presence of ovalbumin. Unpublished data (J. Gautron, S. Solomon, M. Bain, Y. Nys). 1 bar = 100 μm .

tion [54]. This controlled process occurs in a confined space (lumen of the uterus) where ionic concentrations (calcium and bicarbonates) are highly supersaturated [15]. Recently, the investigation of early shell mineralization mechanisms highlighted the importance of the formation of a transient amorphous calcium carbonate mineral (ACC) at the initial stage of eggshell mineralization [55]. The ACC mineral first accumulates on eggshell membranes and on specific nucleation sites (mamillary knobs) (Fig. 7A). ACC deposited around these sites dissolves rapidly, providing a continuous supply of ions to form calcite crystals on specific nucleation sites. These units coalesce to form larger crystals in the mamillary layer, and then during the next rapid growth phase, they form the compact shell palisade layer characterized by columnar crystals with a preferred orientation (Fig. 7A). Calcite crystals result from aggregation of ACC particles that support rapid mineralization of the eggshell and there is evidence that this non-crystalline form of calcium carbonate is present throughout the various phases of shell formation [55]. During these distinct phases, matrix proteins play a key role to stabilize this transient form of calcium carbonate [55] but also influence the selection of the calcite polymorph into which it is ultimately converted and the preferential orientation of calcite crystals in the eggshell [53,56]. This interaction leads to the eggshell ultrastructure and its associated mechanical properties [53,54]. Many efforts are driven to identify and characterize the role of eggshell matrix proteins in the eggshell biomineralization process. Ovalbumin/Serpinb14 was the second protein and the first eggshell serpin identified in the shell matrix [57]. Its presence in the mamillary bodies of decalcified shell was confirmed by immunohistochemistry, indicating that ovalbumin is present during the initial phase of shell formation and becomes incorporated into the protein matrix of the mamillary bodies [57]. The numerous eggshell proteomics studies performed in the last decade, widely confirmed the presence of ovalbumin in eggshell as an abundant protein and identified 13 additional serpins in this biomineral [17–21,23,58].

Ovalbumin/Serpinb14 is believed to play a crucial role in calcium carbonate formation and ACC stabilization [59,60]. Calcium binds to ovalbumin and this accumulation creates a nucleation center for the minerals [60]. Calcium ions are bound to the protein by complexation *via* acidic groups leading to protein structural rearrangements [59]. The calcium cations are the starting points for the subsequent formation of ACC nuclei, which then undergo a series of transition phases to the stable crystalline polymorphs [59]. In a recent study, ovalbumin was reported as a major protein at all key time events of shell mineralization. Furthermore, ovalbumin is overabundant when larger calcite crystal units are growing on the seeding sites of shell (mamillary knobs) [18] and it controls both calcite crystal morphology and size (Fig. 7B and C).

Serpinf2, was identified as a protein of intermediate [23] or major abundance in the shell [18]. Serpinb14b, Serpine2, Serpinf1 and Serpini1 are eggshell matrix protein with intermediate abundance. Serpinb14c, Serpina8, Serping1, Serpinc1, Serpind1 are present in the shell at low abundance or intermediate abundance [18,23]. The remaining 4 eggshell serpins (Serpina6, Serpina1, Serpina4 and Serpinb14c) were identified in very low concentration in the shell. The function of all these serpins has not been explored yet. As potential antiproteases, they could participate in controlling the calcification process by limiting proteolytic degradation [61,62]. Other eggshell serpins such as the antibacterial Serpinb14c could also participate in egg defense [35] within the eggshell and/or during the process of its formation. It is also noticeable that several of these eggshell serpins are actually glycosaminoglycan binding proteins (Serpina14c, Serpind1, Serpinc1, Serpine2). Considering that eggshell matrix contains numerous glycosaminoglycans including keratan sulfate, chondroitin sulfate, hyaluronan and heparan sulfate [63], these heparin-binding proteins may also trigger the interaction with the mineral phase and/or the other eggshell proteins.

surface of both serpins is shown and colored according to values of electrostatic potentials (blue: positive charges; red: negative charges). The cluster of positive charges in OVAX corresponding to the putative heparin-binding site is surrounded by a black circle. This cluster is not present in ovalbumin nor in OVAY despite their high sequence identity with OVAX. Atomic coordinates of ovalbumin (1OVA) and those of OVAX model were obtained by comparative modeling based on ovalbumin structure using Swiss-Model server (swissmodel.expasy.org). The figure was prepared with PYMOL software.

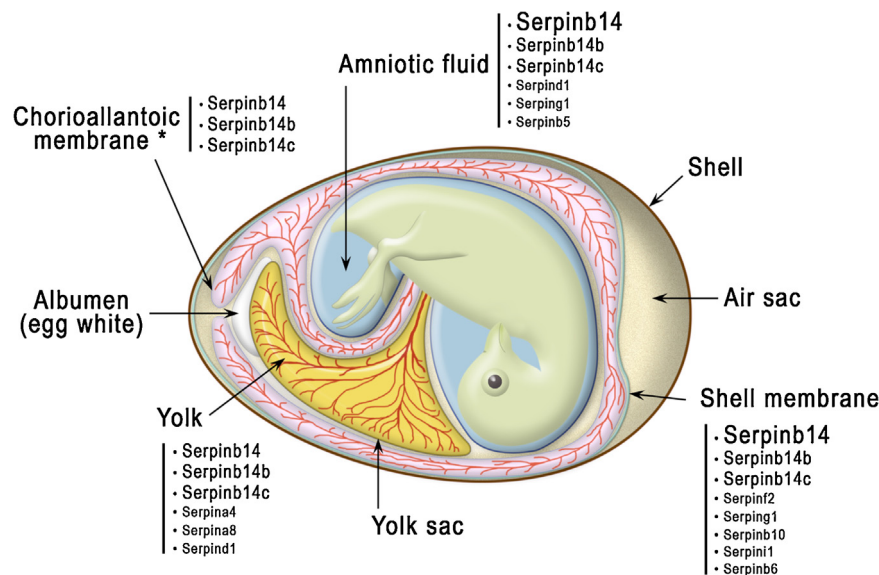


Fig. 8. Schematic representation of a fertilized egg at day 16 of incubation and serpins identified in each egg structure during chicken development. For each compartment, the size of the font roughly indicates the relative quantity of one serpin to another. Bigger letter corresponds to abundant to very abundant proteins, smaller letters correspond to low abundant proteins and normal letters to proteins with intermediate abundance. Note that compartments are not comparable, as the relative quantity intimately depends on the intrinsic composition of each compartment. *Data related to the relative abundance of serpins in the chorioallantoic membrane were not available [71]. Serpins found in amniotic fluid correspond to egg white proteins that presumably transfer to amniotic fluid from day 11 onwards (the composition of egg white remains globally unchanged during the first half of incubation [48]). Data were extracted from [71] (shell membrane and chorioallantoic membrane), [76] (yolk), and [22,28,52] (egg white).

2.5. Serpins in extraembryonic tissues

During the 21-day incubation, the extraembryonic structures that include the amniotic, chorioallantoic and yolk sacs, are essential during embryonic development (Fig. 8). Amniotic sac appears early in the development and embraces the embryo to protect it from mechanical shocks, dehydration and adhesion [64]. In parallel, the allantois expands from the hind gut of the embryo at day 3 of the development (E3) and fuses with the chorion, an extraembryonic membrane lying under the eggshell membranes (Fig. 8). The close contact between the chorioallantoic membrane (CAM) and the eggshell allows oxygenation of the embryo, as well as calcium intake for its skeletal development [64]. This structure also provides a reservoir for disposal wastes produced by the embryonic metabolism, some of its components being reabsorbed by the CAM and used by the embryo for its growth. The yolk sac begins to form from the embryo's gut and encloses the yolk during incubation while the vitelline membrane is disrupted (Fig. 8). This resulting membrane supports yolk nutrients digestion and their transport through the blood system to the embryo [65,66]. Both yolk sac and CAM support angiogenesis. Meanwhile, extraembryonic fluids are transferred from one compartment to another during incubation. Thus, egg white and its components are moving to the amniotic sac from E11 onward, and are orally absorbed by the embryo [64] before reaching the yolk sac [67].

Except ovalbumin which has been detected in many extraembryonic and embryonic tissues, there is little information related to the analysis of serpins in the various structures and fluids of incubated eggs.

2.5.1. Amniotic fluid

Given that egg white transfers into the amniotic sac, ovalbumin (Serpib14) and other egg white serpins are assumed to be found in the amniotic fluid from day 11 onward [68], in the embryonic serum and organs [49]. The antimicrobial properties of OVAX (Serpib14c) could protect the embryo during its development [35], but the impact of the changing environment (i.e. transfer to the amniotic fluid) on its properties has not been explored yet. As regards

to the Serpib14b, its role in embryonic development is still not known. The plasma protease C1 inhibitor/Serping1 and heparin cofactor II/Serpind1 as egg white proteins are supposed to be similarly transferred into the amniotic fluid (Fig. 8). The former has been detected in the woman amniotic fluid [69] and has even been described as synthesized by the amnion [70].

2.5.2. Chorioallantoic membrane and allantoic fluid

Exhaustive analysis of allantoic fluid using proteomic tools has not been investigated yet whereas the proteome of the CAM allowed the identification of three serpins: ovalbumin (Serpib14) and its related protein Serpib14b (OVAY), and Serpib14c (OVAX) [71]. Serpib14 has been found in the chorioallantoic fluid from E6 to E12 [68] and in the CAM and the blood at E19 [71]. Serpib14c and Serpib14b have also been detected in the CAM at E19 [71], but further studies are needed to identify their origins, even if the albumen – amniotic fluid – gastrointestinal tract – chorioallantoic fluid – CAM is assumed to be the main route.

2.5.3. Eggshell membranes

During the embryonic development, there is a global enrichment in serpins' amount in the eggshell membranes (ESM). Serpib14, Serpib14b, and Serpib14c has been described as higher in abundance in the ESM of fertilized eggs compared to the ESM of unfertilized sample all along the incubation. However, Serpib14c and Serpib14b have not been detected in the embryonic blood at E19, which suggests a local expression by the chorioallantoic membrane or a gradual solubilization from the upper part of the eggshell. Serpib10 was found to be enriched in the ESM from fertilized eggs at E3. This serpin is mainly known as a nuclear protein converting DNA into a compact transcriptionally inert heterochromatin [72]. It has been shown to be regulated during development to accumulate in adult chicken erythrocyte nuclei [73] and could also be involved in the chicken host defense [74]. In contrast, neuroserpin/Serpini1, plasma protease C1 inhibitor/Serping1 and alpha2-antiplasmin/Serpif2 were only increased in the third part of the development just before pipping (E19). This distribution implies that each serpin is likely to have a specific role in the ESM

during incubation. Serpini1 is involved in neuronal development and synaptic plasticity, and is restricted to the nervous system of the chicken [75]. The biological significance of its presence in the ESM at E15 remains unclear. Of the eight serpins identified in the ESM during embryonic development, Serpinb6 is the only one to decrease in abundance. Its role in this compartment is not known to date.

2.5.4. Yolk and yolk sac

Ovalbumin/Serpinb14 has been found in the yolk at E18 [49]. It has been proposed that this protein could be digested by enzymes of the egg yolk to release amino-acids and some specific peptides with additional biological activities (antioxidant, antihypertensive etc.) [8].

A small amount of angiotensinogen/Serpina8 has been found in the yolk sac at E0 and decreases until E12 [76]. The protein is expressed by the chicken yolk sac membrane from E2 onward [77], indicating a potential function of the protein in the regulation of homeostasis and primitive erythropoiesis in the yolk sac. Moreover, some have shown that targeted deletion of the genes encoding SERPINA8 produces specific renal abnormalities in mammalian embryos suggesting a crucial role of SERPINA8 in kidney development [78]. Plasma protease C1 inhibitor/Serping1, heparin cofactor/Serpinb1, and alpha-1 antitrypsin/Serpina1 have been detected in very small amount in the yolk of unfertilized eggs [25,27], which question their biological significance (§2.1). Serpind1 and Serpina4 have been described at lower abundance in the egg yolk of fertilized eggs after E12 [76], and thus, might be used by the embryo or the extraembryonic structures during this period.

3. Conclusions

Out of the 27 serpins identified in the chicken genome, only 15 have been detected in the egg. Their localization in the different egg compartments suggests a specific role either during egg formation or during embryonic development. The biological function of most egg serpins is still unknown but the available data suggests that they participate in tissue remodeling and angiogenesis (folliculogenesis, development of yolk, amniotic and allantoic membranes), egg defenses (antibacterial serpins, eggshell serpins) and nutrition (as a source of amino-acids). Amongst the 15 serpins present in the chicken egg, chicken clade B ovalbumin (Serpina14) and its related proteins (Serpina14b, Serpinb14c) retain much interest as these serpins are found exclusively in the egg and as their functions remains largely unknown. This observation suggests that Serpinb14 members might have contributed to evolution of egg laying species, similarly to egg yolk vitellogenins, which have been lost in mammals, concomitantly with the development of placentation in eutherian and marsupial animals [79]. Additional evidence from other species laying eggs would be very informative to strengthen this hypothesis/conjecture. All three Serpinb14 paralogs display subtle conformational and physicochemical differences, which may affect their respective activity. Uniquely in the serpins world, ovalbumin is converted to a heat-stable form while migrating to the extraembryonic fluids and embryonic organs where it recovers as an uncleaved protein. This observation does not actually support its unique role as a source of amino-acids and suggests some more direct function on developing organs. Ovalbumin also contributes to shape eggshell ultrastructure by interacting with the mineral phase. Comprehensive and functional analyses of egg serpins in embryonic and extraembryonic tissues is lacking to date. This review highlights that chicken egg serpins offer multiples axes of research in the field of developmental biology.

Conflict of interest

All authors declare no conflict of interest.

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